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Direct fractionation of proteins in particle-containing feedstocks by a filter paper pieces-based DEAE-cellulose column chromatography Rapid, robust and low-cost capturing procedure for protein

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Abstract

Filter paper pieces-based (FPB) DEAE-cellulose was prepared for direct fractionation of proteins in particle-containing feedstocks. FPB DEAE-cellulose has a protein binding capacity equivalent to that of commercially available DEAE-cellulose. Crude extracts from porcine intestine and kiwi fruit pulp, which were unmanageable by commercially available chromatographic media due to rapid clotting, could be directly fractionated with FPB DEAE-cellulose column. In addition, effluents from an FPB DEAE-cellulose column were extensively clarified. The present approach can be used as a rapid, robust and low-cost capturing step for protein from particle-containing feedstocks.

Keywords: Filter paper pieces-based DEAE-cellulose; Stationary phases, LC; Fruits; Proteins; Collagen

1. Introduction

Protein purification is one of the most important steps in life science and biotechnology. Liquid chromatography has been used extensively for this purpose. Recent advances in chromatographic techniques enable the purification of the protein of interest with high resolution [1,2]. However, crude extracts from animal, plant, fermentation broth and cell culture media may contain cell debris, lipids, polysaccharides, pigments and so on. Application of such crude extracts to a liquid chromatographic column may result in severe operational problems. In such cases, an additional clarification step is necessary to make the extracts more manageable for

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subsequent downstream processing steps. In some cases, such crude extracts may be simply clarified by filtration. However, for the examples in the present paper, filtration has often suffered from rapid clotting by passing through crude extracts. In such cases, preliminary fractionation based on selective protein precipitation using organic sorbents or salts has been performed to clarify and concentrate the protein of interest [1,3]. Addition of such a tedious procedure may decrease recovery of final product and increase processing cost.

In the present communication, we are introducing a filter paper pieces-based (FPB) DEAE-cellulose column chromatography which can directly fractionate protein in particle-containing feedstocks.

2. Experimental

2.1. Materials

Commercially available filters and anion-exchange adsorbents used in the present study are listed in Table 1. They were pretreated and used according to their instructions.

A disposal polypropylene column (1.25 cm I.D.) with 10 ml of sample reservoir and an Econo column (10×2.5 cm I.D.) were purchased from Bio-Rad Labs. (Hercules, CA, USA). C10/10 and 16/70 glass columns were from Pharmacia Biotech (Uppsala, Sweden). Peristaltic pump and pressurizing solvent delivery system were obtained from Atto (Tokyo, Japan) and Dionex (Sunnyvale, CA, USA), respectively.

2.2. Preparation of FPB DEAE-cellulose

The DEAE group was introduced into cellulose fibers from filter paper pieces by the method of Peterson and Sober [4] with slight modifications. Filter paper (16 g; Whatman No. 5) was torn into small pieces (ca. 1×1 cm) by hand. The pieces were boiled in 1 l of distilled water for 15 min and then vigorously mixed in a Warring blender at top speed for 3 min and air-dried. The resultant pulp was put in a 2-l glass jar and mixed with 200 g of 20% (w/w) of NaOH and kept for 30 min at room temperature with occasional stirring using a glass rod. The content was transferred into a 1-l flask with cooling tower and mixed with 2-chlorotriethylamine·HCl solution (35 g dissolved in 45 ml of water) and reacted in a boiling water bath for 2 h with occasional stirring. Reaction was terminated by adding 200 ml of 2 *M* NaCl. The resultant FPB DEAE-cellulose was washed on a glass filter with cold 1 *M* NaOH and 1 *M* HCl in turns until the filtrate became colorless (3–4 times). Then it was re-suspended in 0.1 *M* NaOH and left overnight. FPB DEAE-cellulose was washed thoroughly with distilled water on a cotton cloth and dehydrated with ethanol and air dried. Dry-form FPB DEAE-cellulose was suspended in a suitable buffer using a Warring blender. Small particles generated by vigorous stirring were removed by squeezing in a cotton cloth and then re-suspended by gentle shaking in equilibrium buffer and packed in a suitable column.

2.3. Characterization of DEAE-celluloses

FPB DEAE-cellulose and Whatman DEAE-cellu-

Table 1
List of filters and anion exchangers used in the present study

	Commerical name	Abbreviation	Supplier	Pore (μm)	Diameter (mm)	Material
Filters	GD/X		Whatman	0.45[a]	25	
	C300A		Advantec	3.0	25	
Anion exchangers	Express-Ion Exchanger D	EXP	Whatman			Cellulose
	DEAE-Cellulofine AM	CEF	Seikagaku			Crosslinked cellulose
	DEAE-Toyopearl 650C	TSK	Tosoh			Polymethacrylate
	DEAE Sepharose Fast Flow	SEF	Pharmacia			Crosslinked agarose

[a]Containing prefiltration stack with 10- and 0.7-μm pores.

lose (Express-Ion Exchanger D) were hydrated and directly observed with a Hitachi S-2460N (Hitachi, Japan) scanning electron microscope in natural SEM mode.

Protein (bovine serum albumin, BSA) adsorption and ion-exchange capacities of FPB DEAE-cellulose were estimated by the method reported previously [5].

Mechanical stability of FPB DEAE-cellulose was evaluated by monitoring the pressure drop and the height of gel bed on the glass column (1.6 cm I.D.) packed with the FPB DEAE-cellulose to 20 cm height, to which 0.1 *M* sodium chloride solution was delivered by a peristaltic pump.

2.4. Preparation of particle-containing feedstocks

As a model of particle-containing feedstocks which require additional clarification step for conventional column chromatography, porcine intestinal pepsin-solubilized collagen (PSC) and kiwi fruit extract were prepared.

PSC was prepared from porcine intestine by the method of Sato et al. [6] with slight modifications. Minced intestine was pre-treated with cold 0.1 *M* NaOH to solubilize non-collagenous proteins and exclude the effects of endogenous enzymes on collagens [6]. Insoluble collagenous mass was solubilized by limited pepsin-digestion in 0.01 *M* HCl at 5°C overnight at an enzyme substrate ratio of 1:100 with occasional stirring. Solubilized collagen (PSC) was harvested by centrifugation at 10 000 *g* for 20 min. This extraction procedure was repeated three times. A 2-l volume of PSC solution was mixed with 600 ml of 10 *M* urea containing 0.25 *M* NaCl and its pH adjusted to 8.2 by adding 1 *M* Tris base and then mixed with distilled water to 3 l. Before fractionation with an anion-exchange column, it was re-centrifuged at 10 000 *g* for 20 min. Milk-white turbid supernatant was obtained and referred to crude PSC.

Crude extract of kiwi fruit (*Actinidia chinensis*) was prepared by the method of Sugiyama et al. [7]. Briefly, kiwi fruit juice was mixed with an equal part of 0.01 *M* sodium citrate buffer, pH 5.5, containing 0.001 *M* EDTA and dialyzed overnight against the same buffer. The dialysate was centrifuged at 10 000 *g* for 20 min. Green turbid supernatant was obtained and referred to crude kiwi fruit extract.

2.5. Anion-exchange column chromatography of particle-containing feedstocks

The commercially available anion exchangers as listed in Table 1 and the FPB DEAE-cellulose were packed in polypropylene columns (1.25 cm I.D.) to 10 cm in height. These columns were equilibrated with 0.04 *M* Tris–HCl buffer, pH 8.6, containing 2 *M* urea and 0.05 *M* NaCl or 0.01 *M* sodium citrate buffer, pH 5.5, containing 0.001 *M* EDTA for fractionation of the crude PSC and kiwi fruit extract, respectively. Ten ml of the crude extracts were applied to the sample reservoir of columns. In some cases, the crude extracts were delivered to the glass columns (10×1 cm I.D.) packed with the anion exchangers by nitrogen pressure at 5 p.s.i. or using a peristaltic pump. Volume of effluent from the columns was monitored in suitable intervals. Adsorbed proteins to FPB DEAE-cellulose were eluted by the buffer containing NaCl (0.5 *M*) or ammonium sulfate (1.0 *M*).

2.6. Other analytical methods

Viscosity of the crude PSC was evaluated by a Viscositator VT 03 (Lion, Tokyo, Japan) with a No. 4 rotor at 62.5 rpm. Results were expressed as mPa/s.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli [8]. Protein bands in gel were developed with Coomassie Brilliant Blue R250. The staining intensity of protein bands was evaluated by using a Master Scan 486 (Scanalytics, Billerica, MA, USA).

Protease activity of kiwi fruit was estimated according to the method of Hatakeyama et al. [9] using succinyl casein as substrate.

Protein content in kiwi fruit extract was estimated by the method of Bradford [10] using the Bio-Rad dye reagent.

Turbidity of effluents from columns was evaluated by monitoring absorbance from 220 to 700 nm or at 450 nm.

3. Results and discussion

3.1. Characteristics of FPB DEAE-cellulose

As shown in Fig. 1, commercially available fast-flowing DEAE-cellulose (Express-Ion Exchanger D, Whatman) consisted of short fibers of 60–130 μm in length and 18–20 μm in width. On the other hand, FPB DEAE-cellulose consisted of long (>1 mm) fibers. Thin (2–4 μm) fibers were branched from thick (10–20 μm) fibers.

One gram of the dry FPB DEAE-cellulose gave a volume of 10.7 ml in the swollen state with distilled water. As shown in Fig. 2, FPB DEAE-cellulose has three types of ionic groups, as commercially available ones have [5]. The ion-exchange capacity of FPB DEAE-cellulose was 0.05 mequiv./ml of gel. This value is approximately half of commercially available DEAE-celluloses [5,11]. On the other hand, the protein adsorption capacity of the present gel (60 mg BSA/ml of swollen gel) corresponds to that of the commercially available fast-flowing anion exchangers based on cellulose fiber [11].

As shown in Fig. 3, the gel bed of FPB DEAE-cellulose packed in a column (50×1.6 cm I.D.) remained the initial height by elution of salt solution

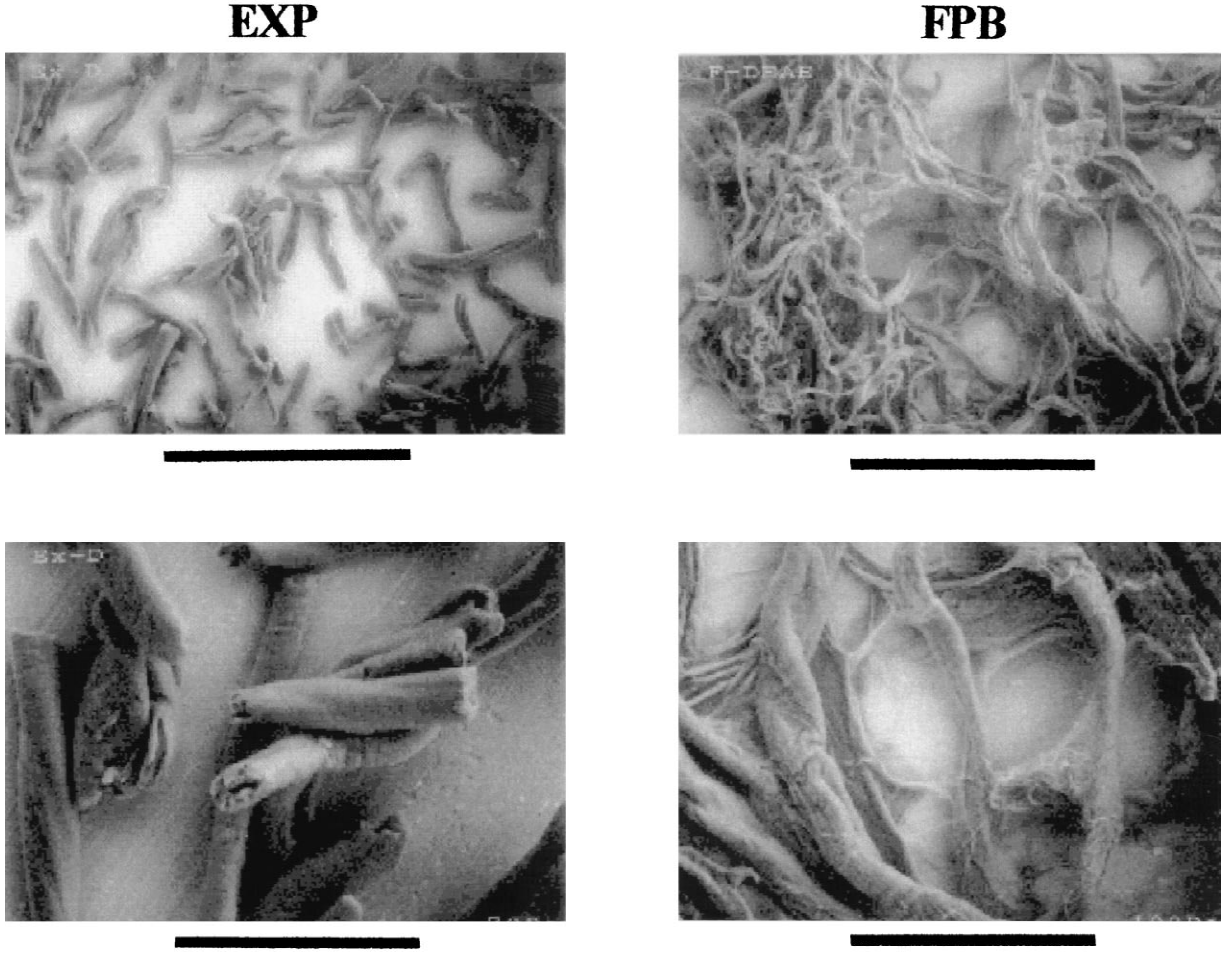

Fig. 1. Appearance of FPB DEAE-cellulose and Whatman Express-ion exchanger D (EXP) celluloses. Rehydrated DEAE-celluloses were observed directly by natural SEM mode. Magnification, ×100 (upper row) and ×500 (lower row). Bars represent 500 and 100 μm, respectively.

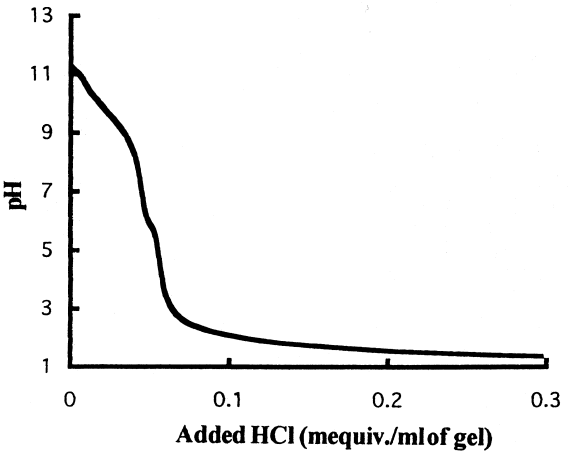

Fig. 2. Titration curve of FPB DEAE-cellulose measured in 0.5 *M* sodium chloride with 0.5 *M* hydrochloric acid.

below a flow velocity of 5 cm/min. Pressure drop by elution of salt solution was also equivalent to the reported values estimated by using a shorter column (15×1.6 cm I.D.) packed with commercially available fast-flowing anion exchangers [5].

3.2. Elution of particle-containing feedstocks from anion-exchange columns

Crude PSC (0.25 mg/ml) showed high viscosity (15 mPa/s), corresponding to 60% of aquatic glycerin solution at 25°C, and was a turbid suspension even after centrifugation at 10 000 *g* for 30 min. Only a negligible amount of crude PSC could be clarified by the filter membranes listed in Table 1, because of rapid clotting. When 10 ml of the crude PSC was applied to the reservoir of columns (10× 1.25 cm I.D.) packed with the commercially available anion exchangers as listed in Table 1, rapid clotting occurred (Fig. 4A). No significant increase of elution volume was obtained by pressuring columns with nitrogen gas at 5 p.s.i. (data not shown). In addition, application of the crude PSC to an empty column also resulted in rapid clotting at endpiece filter of 50 μm. Therefore, the technique using expand beds of adsorbents, which was introduced for purification of proteins from feedstocks containing particles [12], could not be used for fractionation of the present preparation due to clotting at the endpiece filter. Thus only negligible amount of the crude PSC could be fractionated by the columns packed with the commercially available fast-flowing type of anion-exchangers. Therefore, multistep selective salt fractionation was performed for fractionation of collagen molecules in crude PSC before final chromatographic purification [13,14]. On the other hand, the crude PSC was eluted from the FPB DEAE-cellulose column with a sufficient flow-rate (Fig. 4A). As shown in Fig. 5A, the effluent from the FPB DEAE-cellulose column was transparent, indicating that effective clarification occurred. Suspended particles might be trapped in the three-di-

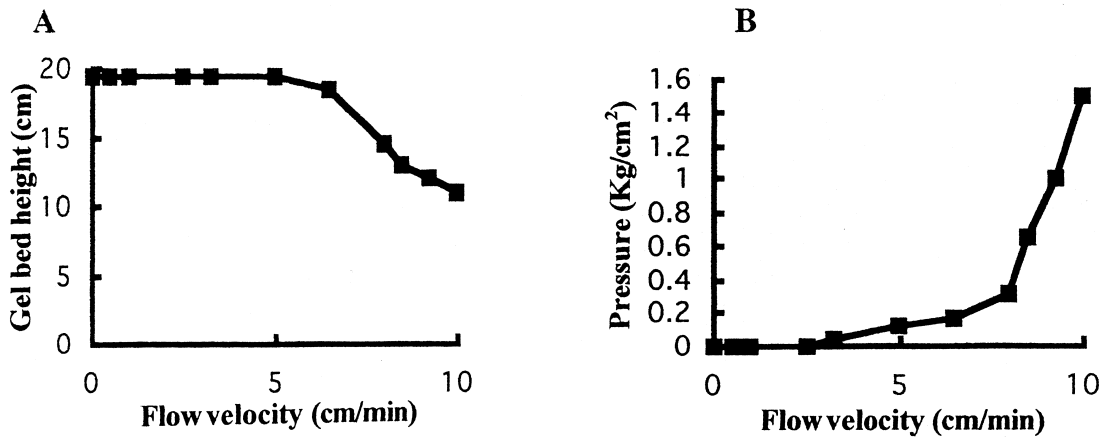

Fig. 3. Effect of flow velocity of 0.1 *M* NaCl on the gel bed height (A) and pressure drop (B) for the column (20×1.6 cm I.D.) packed with FPB DEAE-cellulose.

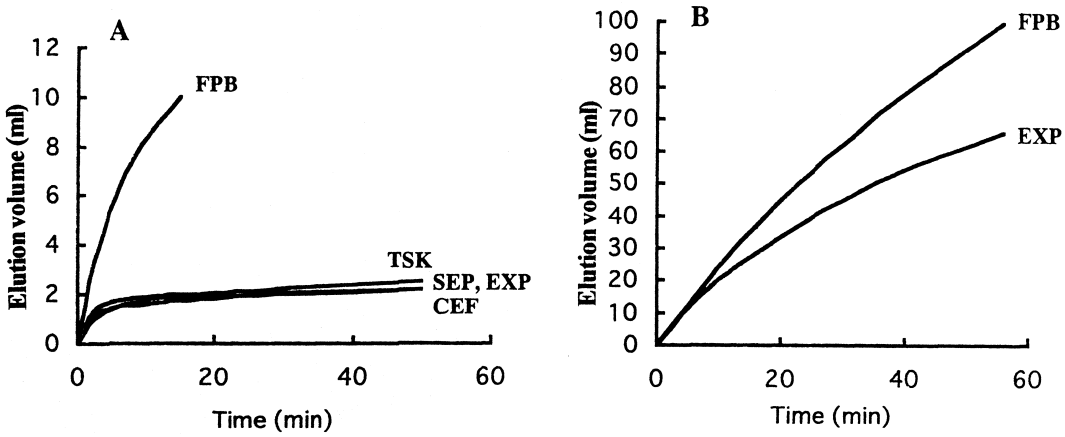

Fig. 4. flow-rate of crude PSC (A) and kiwi fruit extract (B) from the columns (10×1.25 cm I.D.) packed with commercially available adsorbents and FPB DEAE-cellulose. See abbreviations for Table 1.

mensional long entangled fibers of FPB DEAE-cellulose.

Crude kiwi fruit extract (0.47 mg of protein/ml) was a green turbid solution even after centrifugation at 10 000 *g* for 30 min. Filtration of crude kiwi fruit extract resulted in rapid clotting. When the crude kiwi fruit extract was applied directly to the commercially available DEAE-cellulose column, the effluent became turbid green with increase of elution volume (Fig. 5B) and finally clotting occurred, indicating deterioration of gel bed. An additional calcification step based on ammonium sulfate precipitation or batch adsorption to DEAE-cellulose was then used before column chromatographic purification of kiwi fruit protease [15–18]. On the other hand, the flow-rate from the FPB DEAE-cellulose column was

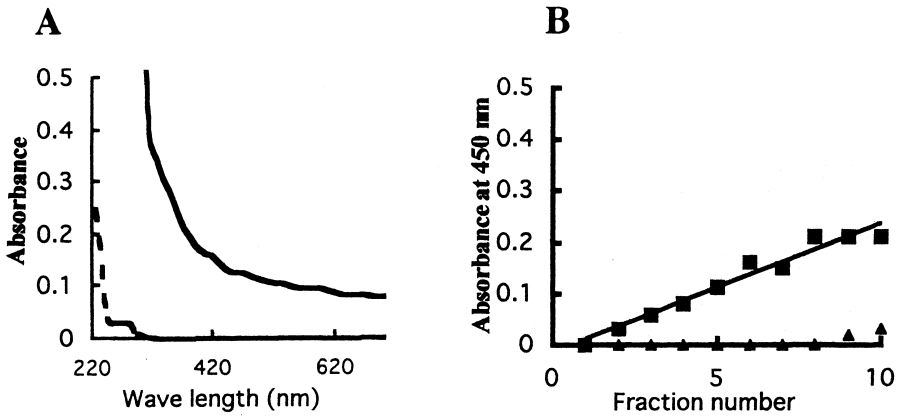

Fig. 5. Clarification of the crude PSC (A) and kiwi fruit extract (B) by passing through the columns packed with DEAE-celluloses. (A) Spectrum of crude PSC before (solid line) and after passing through FPB DEAE-cellulose column (broken line). (B) Turbidity of the effluents from the columns packed with FPB DEAE-cellulose (triangle) and Express-Ion Exchanger D (square). Each fraction volume was 10 ml for kiwi fruit extract.

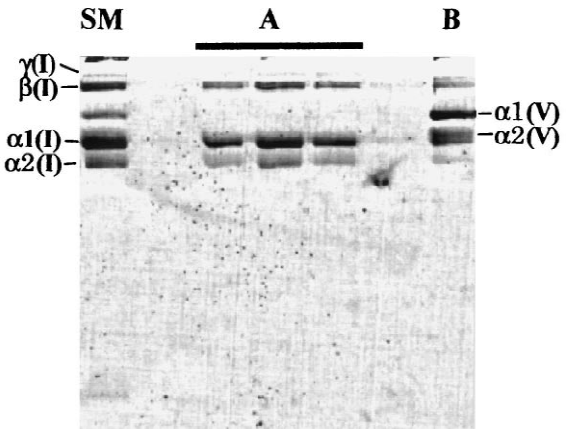

Fig. 6. Fractionation of porcine crude PSC by FPB DEAE-cellulose column chromatography. SM, starting material. (A) Non-adsorbed fraction, (B) adsorbed fraction eluted with 0.5 *M* NaCl. Subunit compositions of type I and V collagens are as follows: Type I, $[\alpha1(I)]_2\alpha2(I)$; and type V, $[\alpha1(V)]_2\alpha2(V)$ [13,14]. β(I) and γ(I) represent cross-linked dimer and trimer of type I α chains.

faster (approximately double) than the commercial one (Fig. 4B), and fractions from the FPB DEAE-cellulose column were transparent (Fig. 5B).

3.3. Fractionation of proteins by FPB DEAE-cellulose column

SDS–PAGE analysis revealed that the crude PSC consisted predominantly of type I collagen with small amount of type V collagen (Fig. 6). One hundred ml (10 ml×10 times) of the crude PSC was applied to the FPB DEAE-cellulose column (10× 1.25 cm I.D.). After washing of the column with 50 ml of sample buffer, adsorbed proteins were eluted with 0.5 *M* NaCl in the buffer. Unadsorbed and adsorbed fractions consisted predominantly of type I and V collagens, respectively, indicating that effective fractionation of collagens in the crude PSC occurred in a few hours. On the other hand, it took nearly a whole week to fractionate crude PSC into type I and V collagens by a conventional procedure based on multistep salt fractionation [13,14]. Recovery of type V collagen by FPB DEAE-cellulose was more than 85% on the basis of staining intensity of the α1(V) chain resolved by SDS–PAGE.

Crude kiwi fruit extract (74.5 mg/200 ml) was applied to the FPB DEAE-cellulose column (10×2.5 cm I.D.) by a peristaltic pump. The column was washed with 100 ml of the sample buffer and adsorbed proteins were eluted with 1.0 *M* ammonium sulfate in the buffer. As summarized in Table 2, most of the protease activity in the extract was adsorbed to the FPB DEAE-cellulose with the equilibrium buffer and eluted with 1.0 *M* ammonium sulfate. The recovery and increase of protease activity by the present study was better than conventional ammonium sulfate fractionation [18]. The protease fraction by the present technique can be fractionated into six isozymes using high-performance anion exchangers for subsequent purification steps [7].

From the above, FPB DEAE-cellulose column chromatography may be a good substitute for tedious selective precipitation techniques for clarification and capturing of the protein of interest from particle-containing feedstocks which are unmanageable for conventional filtration and chromatographic techniques. FPB DEAE-cellulose can be used for batch adsorption of proteins. However, better clarification was achieved by passing through a FPB-DEAE cellulose column than the batch adsorption technique.

Table 2
Summary of fractionation of crude kiwi extract by FPB DEAE-cellulose

	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude extract	200	74.5	5.36	0.07	100	1
DEAE-adsorbed fraction	60	29.5	4.42	0.15	82.5	2.08

It should be also emphasized that FPB DEAE-cellulose can be prepared inexpensively by using commonly available glassware. In addition, no special solvent delivery system is required for the present approach, which can facilitate using many columns for large preparations. We, therefore, anticipate that the present approach has potential for simplifying and shortening purification steps of proteins, and consequently decreasing processing costs, not only at laboratory scale but also at an industrial scale. Further studies on large scale application of the present technique are now in progress.

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